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MOLECULAR MICROBIOLOGY OF GUT BACTERIA: GENETIC DIVERSITY AND COMMUNITY STRUCTURE ANALYSIS*

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Recently developed molecular biology approaches make possible the detailed genetic, taxonomic and ecological examination of microorganisms from various habitats. Animal gut represents one of the most complex microbial ecosystems with a large degree of microbial biodiversity present. Bacteria inhabiting the gut usually play important roles in metabolic transformations of substrates and sometimes, e.g. in ruminants, they make the basis for an obligate symbiosis with the host. Here we discuss molecular microbiology as a strategy for examination of gut bacteria, concentrating on a typical and in such environment dominant group of strictly anaerobic Gram-negative bacteria from the phylogenetic group *Cytophaga*/*Flexibacter*/*Bacteroides*. The bacteria from the genus *Prevotella* are the most abundant Gram-negative bacteria in the rumen and form a distinctive phylogenetic cluster, clearly separated from prevotellas isolated from other ecological niches. They may represent a good choice for a model organism in genetic manipulation experiments and for studies of gene transfer mechanisms taking place in the gut. The molecular tools for detection and monitoring of ruminal prevotellas are discussed.

Keywords: molecular biology, gut bacteria, community structure, genetic diversity

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Modern approaches towards analysis and monitoring of microorganisms in their natural habitats

Understanding of the role that microorganisms play in their natural habitats or ecosystems requires detailed physiological, genetic and ecological knowledge of the specific population as well as individual cells that constitute the population. However, studying the role of specific microorganisms in nature remains difficult, mainly because of the complexity and diversity of natural microbial populations, improper isolation techniques and unsatisfactory cultivability achieved in *in vitro* conditions [1]. Until recently, isolation and capability of growing the microorganisms *in vitro* were the key points in such studies which were inevitably missing large proportions of species and even genera present but uncultivable. In the late 1980's molecular biology approaches were used for investigation of microbial biodiversity. Researchers analyzed parts of microbial genomes directly via cloning and sequencing and thus avoided the isolation/cultivation steps, hoping, that the variability in sequences of the analyzed genes will reflect the variability of the host genomes and therefore microorganisms. The small ribosomal subunit was chosen as the perfect molecular target for such studies, due to its evolutionary chronometer behavior [2].

With the onset of the PCR reaction, which made the whole procedure of amplification of the 16S rRNA genes and subsequent cloning substantially easier, a variety of natural microbial ecosystems was analyzed showing that indeed an enormous number of bacterial species and even genera remained hidden due to our incapacity to isolate and grow them *in vitro*. Subsequently other techniques, mainly based on the modification of the PCR reaction, were developed, which made possible not only the qualitative analysis of the genetic diversity of certain microbial community, but also quantification and monitoring of individual species or groups and thus the dynamics of the total population. Competitive PCR seems to be one of the most efficient, reproducible, and flexible methods for molecular quantification of targeted microroganisms [3, 4]. T-RFLP or terminal restriction analysis is on the other hand a method that makes possible the assessment of changes of the structure of a complex population [5–7] as does PCR-DGGE technique too [8–11]. *In situ* hybridization using fluorescently labeled oligonucleotide probes is another alternative which can be combined with either epifluorescent microscopy [1] or flow cytometry [12] for a faster, automated multifactorial analysis.

Gut microbiology and molecular methods

Microorganisms were known for decades to play immensely important roles as inhabitants of the alimentary tract in animals and humans, especially in animals feeding mainly on plant material. Ruminants for example, developed an intimate symbiotic relationship with the microorganisms living in the foregut, depending entirely on the capacity of their symbiotic partner to degrade the main substrate i.e. cellulose and hemicelluloses [13–15]. A number of species and genera were identified and studied, however rumen microbiologists were aware that despite of the development of rigorous anaerobic cultivation methods and suitable media, at least some functionally important groups may not have been recovered yet [14]. The level of knowledge was even lower in other gut models, somewhat better in human, pig, rodent and interestingly termite gut, but much more scarce in others.

Molecular approaches were used in gut microbiology soon after these have been first applied for examination of less complex ecosystems. Radioactively or fluorescently labeled phylogenetic oligonucleotide probes were used in hybridization experiments first [16, 17] and later these probes and other oligonucleotide sequences were used as PCR primers in straightforward PCR reactions or their variations. Finally, cloning and sequencing of ribosomal genes was performed for analysis of human [18–20], ruminal [21–23], porcine [24] as well as termite microbial biodiversity [25, 26]. *In situ* hybridization techniques are now used widely for detection, enumeration and monitoring of certain bacterial species or groups in gut samples [17, 27–29]. First attempts to use flow cytometry in combination with *in situ* hybridization in order to monitor ruminal bacteria were reported too [30].

Rumen and the bacterial genus *Prevotella*

Gram-negative bacteria, identified as members of the genus *Prevotella* belong to a phylogenetic group *Cytophaga*/*Flexibacter*/*Bacteroides* (CFB) [31]. Together with members of the genus *Bacteroides*, they usually constitute one of the dominant bacterial populations inhabiting anaerobic parts of animal gastrointestinal tract. Anaerobiosis usually requires complex structure of the gastrointestinal tract, but is essential for growth of *Bacteroides* and *Prevotella* spp. Interestingly, a ribotype moderately related to *B. acidofaciens* and *B. eggerthii* was recently discovered also in a very simple, seemingly aerobic, part of the gut of common woodlouse *Porcelio scaber* [32]. It seems therefore likely that small anaerobic niches

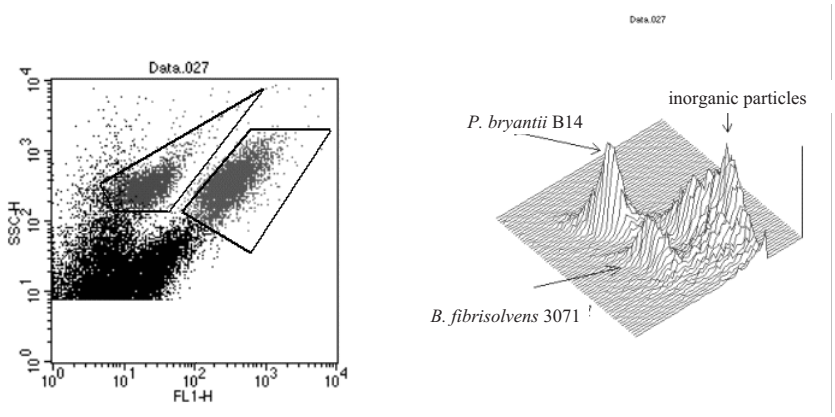

Figure 1. Flow cytometric analysis of mixed cultures *P. bryantii* and *B. fibrisolvens*. The discrimination of bacterial cells from different species and from inorganic particles can be seen on two-dimensional dot plot (left) and two-dimensional histogram (right) [30]

can be established also in prevailingly aerobic gut segments and that strictly anaerobic bacteria can survive in what looks at the first glance a hostile i.e. aerobic environment. The later is in agreement with recent findings showing that methanogenic archaea inhabiting the termite gut live in close proximity or partially even within the aerobic zone of the gut even though they are strict anaerobes [33].

Whereas *Bacteroides* spp. prevail in the hindgut of monogastric animals, prevotellas are recognized as one of the most abundant groups of ruminal microorganisms. They were first identified in 1950's and classified as members of the species *Bacteroides ruminicola* [34], but have been later transferred to the genus *Prevotella* by Shah and Collins [35]. Several authors have demonstrated the abundance of the ruminal prevotellas with traditional enumeration methods and in certain cases even up to 70% of isolates grown on nonselective medium were identified as prevotellas [36].

These findings were confirmed recently by molecular studies, using restriction enzyme profiling of PCR amplified 16S rRNA genes. The prevotellas accounted for between 12 and 62% of total eubacterial 16S rDNA from rumen fluid samples of sheep and cow [37]. That all made the genus *Prevotella* potentially the most interesting ruminal Gram-negative bacteria for gene transfer and manipulation studies. Unfortunately, only few cryptic plasmids were discovered in ruminal *Prevotella* isolates that could be used as the basis for the construction of efficient shuttle vectors [38] and no transposable elements from these bacteria have been described yet. Tetracycline resistance genes were found which could be used as markers in gene transfer studies [38], but on the other hand strong deoxyribo-

nuclease activities were disclosed, hampering the progress [39, 40]. A temperate bacteriophage was identified [41], however, the 16S rRNA sequence analysis of the host strain AR29 showed that it was actually not a true *Prevotella*, but rather a member of the *B. fragilis* group (unpublished). Despite of the lack of the genetic tools available, several genes of ruminal prevotellas have already been cloned and analyzed [42–50] and some work was done also on the development of the gene transfer systems [51–57]. It is necessary, however, to continue the search for such genetic elements in order to successfully establish a truly useful genetic system for ruminal prevotellas.

Molecular taxonomy and genetic diversity of the ruminal prevotellas

Due to the recognized heterogeneity of the species on the biochemical and genetic level [58–60], the species were split into four separate species named *P. ruminicola, P. brevis, P. albensis* and *P. bryantii* [61]. Several studies investigating the genetic diversity of ruminal bacteria through sequence analysis of directly amplified and cloned ribosomal genes were published recently [21–23]. The first two studies screened the total bacterial population in the rumen, whereas Ramšak et al. [23] focused on the members of the phylogenetic group *Cytophaga*/*Flexibacter*/*Bacteroides* (CFB). It was shown clearly that within the Gram- negative cluster of ruminal bacteria the majority of the ribotypes belong to the CFB phylogenetic group and that the organisms harboring these genes must be members or close relatives of the genus *Prevotella* [21, 22]. It was shown also that the majority of the cloned ribotypes belong to a "supercluster" of so-called "ruminal prevotellas", which are phylogenetically clearly separated from prevotellas isolated from other ecological niches (Figure 2) [23]. The members of this supercluster form several (at least six) taxonomic groups however, most probably on the species level. When the comparison of the ribotypes, obtained directly from the total rumen microbial DNA, with the 16S rRNA sequences from isolated ruminal *Prevotella* strains was done, it became clear that there are already isolated strains available for the majority of defined groups, which were previously identified only by PCR amplification and sequencing.

In bacteria, the genes coding for rRNA are usually linked within one operon in 16S rRNA–23S rRNA–5S rRNA order with internal spacer regions (ISR) between them [62]. A common assumption, that copies of rRNA genes within the same organism are identical, has been questioned recently since several reports described considerable differences in nucleotide sequences between copies of rRNA

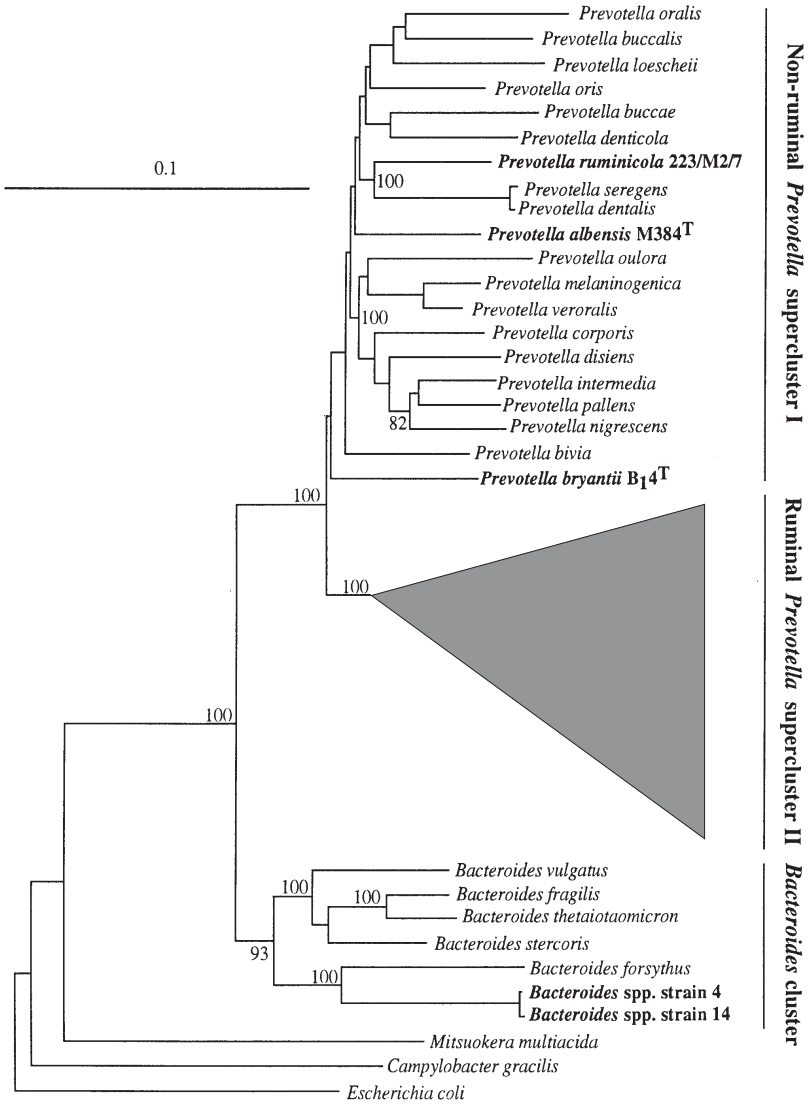

Figure 2. Phylogenetic placement of 16S rDNA sequences from cultivated rumen bacteria belonging to the CFB phylum. The *E. coli* sequence is used as the outgroup for rooting the tree. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. The tree is a modification of Figure 1 from Ramšak et al. [23]

genes in a single organism [63–65]. Such findings are important from phylogenetic as well as functional aspects. To analyze such possible heterogeneities, individual copies of rRNA genes must be cloned and sequenced. Four to six ribosomal operons were found in type strains of ruminal *Prevotella* species and preliminary analysis of some of the cloned genes did not show sequence variations above 2 % (Peterka and Avguštin, unpublished), which sustains the findings from molecular studies mentioned above.

Molecular tools for detection and monitoring of the prevotellas

The construction of useful molecular tools for specific detection and monitoring of microorganisms in their natural environments depends mainly on discovery of appropriate target regions. These are mainly short DNA or RNA sequences for which complementary oligonucleotides can be synthesized and appropriately labeled. Ribosomal RNA sequences are particularly suitable, since a growing cell usually harbors several thousand ribosomes acting as targets in *in situ* hybridization experiments [1]. First oligonucleotide sequences developed for ruminal prevotellas were used as PCR primers [56]. Particularly useful proved to be the broad range primer named BacPre, which was later shown to be very much conserved within ruminal prevotellas. Other broad range oligonucleotide probes, covering a number of taxonomic groups within the CFB phylum were published later [66], however, it was shown that only a minor part of the retrieved ruminal rRNA sequences are homologous with them [23].

Based on the described specific oligonucleotides, competitive PCR systems for specific detection and enumeration of all ruminal prevotellas as well as species *P. ruminicola* and *P. bryantii* are being developed (Figure 3) [67]. The attemepts for enumeration and monitoring of ruminal prevotellas in the rumen of a cow and sheep as well as horse and other monogastric animals are currently undergoing.

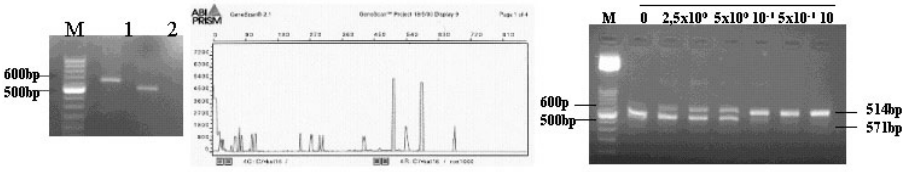

Figure 3. Construction of the internal control for the competitive PCR specific for ruminal prevotellas [61]. The internal control shares the same sequence as the target region, but is slightly shorter due to removing of the middle part through restriction with appropriate endonucleases (left and in the middle). Choosing the correct dilution of the internal control, a series of competitive PCR reactions was performed with serially diluted sample DNA

What can be expected in the near future

With the use of modern molecular approaches, we can realistically expect to gain substantial new information about the microbial community structure inhabiting the animal and human gut. Presumably, new species from this genus will be discovered, whose existence was until now indicated only by direct retrieving and analysis of ribosomal genes. The analysis of the ribosomal gene sequence databases and the large number of available rRNA sequences from ruminal prevotellas and other microorganisms already makes possible the search for species or genera specific sequences which can be utilized for construction of oligonucleotide probes or primers. The use of molecular tools like cPCR, T-RFLP or DGGE-PCR and *in situ* hybridization combined with epifluorescent microscopy or flow cytometry will make possible not only detection and studies concerning spatial distribution of targeted microorganisms, but also rapid, efficient and reliable enumeration which will be the basis of true monitoring of the species or genera status in the given habitat. The role and importance of bacterial genera such as *Prevotella* will finally be elucidated as well as the distribution in various gastrointestinal environments.

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